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Immunological Cross-Reactivity in the Absence of DNA Homology Between *Pseudomonas* Toxin A and Diphtheria Toxin

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The immunodominant determinant of *Pseudomonas* toxin A was shown to cross-react with a normally inaccessible determinant in fragment A of diphtheria toxin. Trypsin-treated diphtheria toxin and fragment A of diphtheria toxin inhibited binding of toxin A antibody to whole toxin A, whereas whole diphtheria toxin did not inhibit this reaction. However, even at the lowest stringency no hybridization was detected between diphtheria *tox* probe and *Pseudomonas aeruginosa* DNA.

Pseudomonas aeruginosa toxin A and diphtheria toxin both inhibit eucaryotic protein synthesis by catalyzing the NAD-dependent ADP ribosylation of EF2 (7, 13). The ADP-ribosyl transferase activities of these two toxins are remarkably similar if not identical (5, 13, 14). Fragment A of diphtheria toxin and a 26,000 to 27,000-dalton, enzymatically active fragment from toxin A have similar Michaelis constants (K_m's) for NAD and EF-2 (8 and 15 μM, respectively) and similar inhibition constants (K_i's) for a variety of analogs. In addition, the ADP-ribosyl transferase reaction catalyzed by fragment A of diphtheria toxin can be reversed by toxin A or its enzymatically active fragment or vice versa (5, 14). These data suggest at least partial homology between these two toxins. Demonstration of immunological cross-reactivity between toxin A and diphtheria toxin would support this contention, but this has not been possible by immunodiffusion analysis (16), enzyme neutralization (13), or cross-neutralization of tissue culture cytotoxicity (18).

We have examined the possibility of homology between toxin A and diphtheria toxin by utilizing a very sensitive solid-phase radioimmunoassay inhibition system (SPRIA inhibition) and DNA-DNA hybridization with DNA from β-tox$^+$ corynebacteriophage (3) and the toxin A$^+$ *P. aeruginosa* strain PA103. Cross-reactivity was demonstrated by using the SPRIA inhibition system. However, even at the lowest stringency no hybridization was detected between diphtheria *tox* probe and *P. aeruginosa* DNA.

MATERIALS AND METHODS

Toxin A was purified as previously described (15). Diphtheria toxin was obtained from Connaught Medical Research Laboratories, Toronto, Ontario, and further purified as previously described (22). Diphtheria toxin was treated with trypsin and dithiothreitol (DTT) as described by Collier and Kandel (8). Fragment A of diphtheria toxin was purified essentially as described by Collier and Kandel (8). Fragment B of diphtheria toxin was purified by the method of Pappenheimer et al. (20). Rabbits were hyperimmunized with toxin A (15), diphtheria toxoid, fragment A of diphtheria toxin, and partially purified fragment B of diphtheria toxin as previously described (17, 22).

Phosphate-buffered saline for radioimmunoassay was Dulbecco phosphate-buffered saline (GIBCO Laboratories, Grand Island, N.Y.). The "filler" diluent contained 10% (vol/vol) fetal calf serum, 0.2% sodium azide, and 0.02% phenol red as described by Zollinger et al. (23).

SPRIA inhibition. The SPRIA inhibition was performed essentially as described by Zollinger and Mandrell (24). Each rabbit serum was first tested for antibody levels by SPRIA against toxin A or diphtheria toxin in round-bottom microtiter plates (Cooke Laboratory Products, Inc., Alexandria, Va.) as described by Zollinger et al. (23). Plates were sensitized with 25 μl of a 50-μg/ml solution of toxin. Goat anti-rabbit immunoglobulin, obtained from Antibodies Inc., Davis, Calif. and labeled by the lactoperoxidase method to a known specific activity (23), was utilized as the secondary antibody. An estimate of the nanograms of antibody per milliliter in each serum specific for toxin A and diphtheria toxin was thereby obtained. The sera were diluted in filler diluent to approximately 100 ng of specific antibody per ml. This dilution of serum resulted in 20 to 30% of the maximum or plateau level of ^{125}I bound.

Inhibition mixtures consisting of 50 μl of diluted antisera (~ 100 ng/ml) and 50 μl of inhibitory antigens at various concentrations were placed in the wells of a separate polystyrene microtiter plate and allowed to react at 37°C for 1 h. Control wells contained filler only (background) and filler plus serum (no inhibitor). After incubation, duplicate 25-μl samples were placed in the toxin-coated wells of the flexible microtiter plate. Binding was allowed to proceed overnight at room temperature, after which the liquid was aspirated and the wells were washed once with filler and five times with phosphate-buffered saline. ^{125}I-labeled goat anti-rabbit immunoglobulin (25 μl) was then placed in each well. Double-strength ^{125}I-labeled goat anti-rabbit immunoglobulin was added to two control wells which did not contain inhibitor to ensure saturation binding to the antitoxin antibody. After incubation, aspiration, and washing, the wells were cut into tubes and counted. Percent inhibition was calculated according to the formula of Zollinger and Mandrell (24): Percent inhibition = 100 − [(mean cpm bound with inhibitor + background)/(mean cpm bound without inhibitor)] × 100. Inhibition experiments were performed three separate times with similar results. Figure 1 is a representative experiment. Values between 50 and 100% inhibition were found to be specific and reproducible in the toxin-antitoxin inhibition system. Zollinger and Mandrell found that background or nonspecific inhibition varied from 0 to 40% depending on the sera they used (24).

RPHA. Reversed passive hemagglutination assay (RPHA) was performed as described by Holmes et al. (10), using immunoabsorbent-purified, sheep antitoxin A antibody-sensitized, formalized sheep erythrocytes (1). The RPHA endpoint represented the smallest quantity of the material tested that caused complete agglutination of sensitized SRBC. Controls as described by Holmes et al. (10) were included in all assays.

DNA/DNA hybridization. DNAs of tox$^+$ corynebacteriophage β-*tsr*-3, a heat-inducible mutant of β-converting phage and of *Corynebacterium diphtheriae* strain C7(−), were isolated as recently described (2).

P. aeruginosa DNA was isolated from cells grown at 37°C with aeration to a concentration of approximately 10^9 cells per ml in the Trypticase soy-yeast extract medium used for *C. diphtheriae*. The cells were pelleted by centrifugation (10 min, 8,000 × *g*), resuspended to 1/10 of their original volume in 5 mM Tris–5 mM disodium EDTA (pH 7.5), equilibrated to 60°C, and lysed by the addition of sodium dodecyl sulfate (0.5% final concentration). The released DNA was sheared and isolated as described for *C. diphtheriae* DNA. The released DNAs were digested to completion with the appropriate restriction endonuclease (*Sal*I and *Bam*HI), electrophoresed for 14 h at 50 V on a 0.5% horizontal agarose gel, and blotted to nitrocellulose filters as described (3). However, the incubation temperature for hybridization was lowered to 65°C, and the prehybridization and hybridization conditions were changed from 2× to 10× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) to achieve different stringency conditions (12). The filters were washed as described except that the wash temperature was 65°C. The first six washes were at 10× SSC, and the final washes were at (i) 0.4× SSC, high stringency; (ii) 2× SSC, intermediate stringency, and (iii) 10× SSC, low stringency. The specific activity of the probe was 32 × 10^6 cpm/μg of DNA, which equaled 10 to 20 ng of DNA per ml of hybridization fluid.

Enzyme assay and neutralization by antibody. Enzyme neutralization assays were performed by incubating toxin A (100 ng; treated with urea and DTT) or fragment A of diphtheria toxin in 10 μl of 5% normal rabbit serum with an equal volume of immune serum for 10 min at 37°C as previously described (9, 13). The ADP-ribose transferase activities of toxin A and fragment A of diphtheria toxin were then measured as described previously (13, 15).

RESULTS AND DISCUSSION

The ability of toxin A, diphtheria toxin, diphtheria toxin treated with trypsin and DTT, and fragment A of diphtheria toxin to inhibit the reaction between rabbit antitoxin A (50 ng/ml, final concentration) and toxin A is shown in Fig. 1. At final concentrations of 128 μg/ml, trypsin-DTT–treated diphtheria toxin inhibited 90% of the toxin A-antitoxin A binding, fragment A of diphtheria toxin inhibited 84% of the reaction, and diphtheria toxin only inhibited between 20 and 30% of the binding. The inhibition seen with diphtheria toxin was probably nonspecific since inhibition did not increase with increasing concentrations of inhibitor. Trypsin alone as a control gave background levels of less than 20% inhibition (data not shown). In the range of inhibitor concentrations yielding 50 to 100% inhibition, trypsin-DTT–treated diphtheria toxin was as potent an inhibitor as toxin A itself (Fig. 1). Toxin A was a more potent inhibitor than trypsin-DTT–treated diphtheria toxin or fragment A of diphtheria toxin at lower inhibitor concentrations. Concentrations of toxin A as low as 32 ng/ml produced 40% inhibition, and concentrations in the range of 1 to 16 ng/ml produced 20 to 30% inhibition. In contrast, concentrations as high as 2,000 ng of trypsin-DTT–treated diphtheria toxin, or fragment A of diphtheria toxin, per ml produced only 20 to 30% inhibition.

Toxin A and diphtheria toxin therefore share an antigenic determinant which is highly accessible in toxin A but is readily accessible in diphtheria toxin only after the molecule is treated with trypsin and reducing agent. This determinant is probably located in the A portion of the diphtheria toxin molecule, as evidenced by the ability of purified fragment A of diphtheria toxin to inhibit the reaction to the same degree as trypsin-DTT–treated diphtheria toxin (Fig. 1).

It would be tempting to assume that the cross-reactive determinant was located in the enzyme-active site of fragment A of diphtheria toxin since the two toxins have such similar ADP-ribosyl transferase activities. The rabbit antiserum we used to demonstrate the cross-reactive determinant was able to neutralize toxin A enzy-

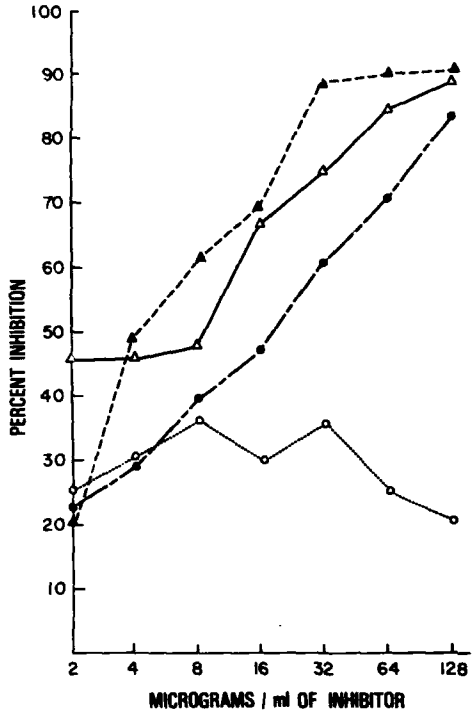

FIG. 1. SPRIA inhibition of rabbit antitoxin A (50 ng/ml, final concentration) binding to toxin A, utilizing various concentrations (final) of either toxin A (△), diphtheria toxin (○), diphtheria toxin treated with trypsin and DTT (▲), or fragment A of diphtheria toxin (●) as inhibitors.

matic activity but was unable to neutralize the enzyme activity of fragment A of diphtheria toxin (data not shown). This finding is consistent with those of Iglewski and Kabat (13), who were unable to neutralize the enzyme activity of fragment A of diphtheria toxin with pony sera which neutralized toxin A enzyme activity. If the cross-reactive determinant is in the enzyme-active site of fragment A of diphtheria toxin, lack of enzyme neutralization by antibody against this site could be explained if the antibody had lower affinity for the cross-reactive determinant when located in fragment A of diphtheria toxin than when located in toxin A. This hypothesis is consistent with the ability of toxin A to inhibit the SPRIA at much lower concentrations than fragment A of diphtheria toxin.

Alternatively, the cross-reactive determinant may be located in a noncatalytic site. Rittenberg et al. (22) have previously suggested the existence of at least two independent antigenic regions on fragment A of diphtheria toxin, only one being located in the enzyme-active site of the molecule. We are presently unable to distinguish between these two possibilities on the basis of our data.

TABLE 1. Inability of toxin A to inhibit SPRIA reactions between diphtheria toxin and sera raised against diphtheria toxin antigens

Antigen	Antibody[a]: against	% Inhibition by antigen[b]: DT	DT Tryp-DTT	Toxin A
DT	D toxoid	92	95	5.4
DT	DT-A	89	98	3.7
DT	DT-B	77	98	6.7
Toxin A	Toxin A	14	93	92

[a] SPRIA inhibition was performed as described in the text. Antibodies were at 50 ng/ml, final concentration. Antibodies were obtained by immunization of rabbits with diphtheria toxoid (D toxoid), fragment A of diphtheria toxin (DT-A), or fragment B of diphtheria toxin (DT-B).

[b] Percent inhibition of the antigen-antibody reaction. Inhibiting antigens were at a final concentration of 50 μg/ml. DT, Diphtheria toxin; DT Tryp-DTT, trypsin- and DTT- treated diphtheria toxin.

The SPRIA inhibition technique primarily measures the ability of competing antigens to bind with the highest-titered antibody in the sera specific for those antigens. The antibody in highest titer against toxin A as measured in the solid-phase system was fully inhibited by the same concentrations of trypsin-DTT–treated diphtheria toxin and fragment A of diphtheria toxin as by toxin A itself (Fig. 1). Therefore the immunodominant determinant of toxin A is very similar to the cross-reactive determinant on diphtheria toxin.

In contrast, antibodies against the cross-reactive determinant were not demonstrated when we examined reactions between diphtheria toxin and sera obtained from rabbits immunized with either diphtheria toxoid or fragment A or B of diphtheria toxin. The SPRIA reactions between these sera and diphtheria toxin were completely inhibited by diphtheria toxin and trypsin-DTT–treated diphtheria toxin, but were unaffected by toxin A (Table 1). The highest-titered antibodies in these sera were therefore directed against determinants other than those cross-reacting with toxin A. The cross-reactive determinant, when part of the diphtheria toxoid molecule or in the isolated fragment A of diphtheria toxin, is therefore not as good as immunogen as when found in toxin A. Our data are consistent with the proposal by Pappenheimer et al. (20) that most of the antigenic determinants of fragment A of diphtheria toxin are masked in the intact molecule. Clearly, the immunodominant determinant for rabbits on fragment A of diphtheria

TABLE 2. Immunological cross-reactivity between toxin A and diphtheria toxin and fragment A of diphtheria toxin, by RPHA.

Antigen	RPHA endpoint[a]
Toxin A	0.1
Diphtheria toxin	25.0
Fragment A of diphtheria toxin	10.0

[a] The RPHA endpoint represented the smallest quantity of the material tested that caused complete agglutination of sheep erythrocytes sensitized with immunoabsorbent-purified sheep antitoxin A antibody.

toxin is not the cross-reactive determinant we are describing.

Despite the limited accessibility of the common determinant on diphtheria toxin, direct binding between immunoabsorbent-purified, sheep antitoxin A antibody and diphtheria toxin could be demonstrated by an RPHA capable of detecting 0.1 ng of toxin A (Table 2). A 25-ng sample of diphtheria toxin caused agglutination of the antitoxin A-sensitized sheep erythrocytes in this system, and 10 ng of fragment A of diphtheria toxin caused agglutination.

The cross-reactive determinant demonstrated between toxin A and diphtheria toxin by inhibition and direct binding experiments suggested there may be at least partial homology between the genes for these polypeptides. To define the extent of this homology, we attempted to hybridize DNA from tox$^+$ corynebacteriophage to whole-cell DNA from *P. aeruginosa* PA103. A *Bam*HI restriction endonuclease fragment (3.9 kilobases; 2.6×10^6 daltons) of β corynebacteriophage recently identified as carrying the *tox* gene (3) was hybridized to nitrocellulose filter blots of *Sal*I restriction endonuclease-digested whole-cell DNA from PA103. As a positive control, *P. aeruginosa* DNA and *Bam*HI-digested whole bacteriophage β DNA were mixed in amounts yielding approximate equality in the number of their genomes. To calculate the amount of DNA needed for this, the molecular mass of *P. aeruginosa* chromosome was taken as 2.1×10^9 daltons (21), and that for β phage was taken as 2.3×10^7 daltons (4). A negative control employing *C. diphtheriae* nonlysogenic C7(−) whole-cell DNA digested with *Bam*HI was also run. The stringency requirements for the DNA/DNA hybridizations were varied such that the degree of base pair mismatch allowed in stable duplexed DNA ranged from 10 to 15% at high stringency to 20 to 25% at intermediate stringency and 30 to 35% at low stringency. Stringencies were calculated according to the formula given by Howley et al. (12).

The results (Fig. 2) showed that even at the lowest stringency, no hybridization was detected between the diphtheria *tox* probe and strain PA103 DNA. In the positive control, only the fragment previously identified as the *tox*-bearing fragment of phage DNA hybridized to the probe. This result also demonstrated that the techniques employed could detect a sequence homologous to *tox* if it were present at an equivalent of one copy per bacterial genome copy. The negative control with C7(−) DNA in which no hybridization was seen showed that the probe contained only phage DNA. Similar results (data not shown) were obtained with DNA from *P. aeruginosa* PA01, a second toxin A-producing strain. From these data we conclude that there is no measurable homology between the *tox* gene of *C. diphtheriae* and the DNA of toxin A-producing *P. aeruginosa*.

FIG. 2. DNA/DNA hybridization between the *tox* gene-containing DNA restriction fragment of β corynebacteriophage and whole-cell DNA of *P. aeruginosa* PA103. A, B, and C represent high, intermediate, and low stringency conditions, respectively. Lane 1 contained ca. 2 μg of *Sal* I-digested DNA from *P. aeruginosa* PA103; lane 2 contained the same amount of *P. aeruginosa* DNA plus ca. 0.022 μg of *Bam*HI-digested β-*tsr*-3 phage DNA; and lane 3 contained ca. 2 μg of *Bam*HI-digested whole-cell *DNA* from nonlysogenic *C. diphtheriae* strain C7(−).

The results of the hybridization studies indicate that despite an identity in mode of action and evidence for cross-antigenic reactivity, the genes for diphtheria toxin and toxin A of *P. aeruginosa* are quite distinct in their nucleotide sequences. By way of comparison, DNA homology has recently been demonstrated between the LT-A and LT-B fragments of *Escherichia coli* LT toxin and the A and B fragments of cholera toxin (19). Homology was detected under conditions in which a mismatch of base pairs of greater than 20% was allowed for LT-A, and 36% was allowed for LT-B. Both A and B

fragments of *E. coli* LT toxin and cholera toxin show antigenic cross-reactivity (6), and both toxins share an affinity for similar cell receptors (11).

Two interpretations can be given to our results. The first is that genes for diphtheria toxin and toxin A evolved independently, but that adaptation to a similar substrate resulted in enzymes which share a limited but antigenically active amino acid sequence. Alternatively, the genes for these toxins may have had a common ancestry, but over time lost significant homology. The former interpretation seems most likely, given the fact that homology would have been detected under conditions in which an average of one out of every three base pairs were mismatched. Amino acid sequencing of the toxins or nuceolotide sequencing of their genes will be required before a definitive interpretation of these results is possible.

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The immunodominant determinant of Pseudomonas toxin A was shown to strongly cross react with a normally inaccessible determinant in the A fragment of Diphtheria toxin. Trypsin treated Diphtheria toxin and A fragment of Diphtheria toxin inhibited binding of toxin A antibody to whole toxin A while whole Diphtheria toxin did not inhibit this reaction. However, even at the lowest stringency no hybridization was detected between diphtheria tox probe and Pseudomonas aeruginosa DNA.

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